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Journal of Experimental Research on Human growth & Aging

Key Molecules Involved in Signalling Transduction Pathways of Nasopharyngeal Carcinoma

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Research Article

Volume 2 Issue 1

Received Date: March 13, 2019

Published Date: March 25, 2019

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Abstract

The association of nasopharyngeal carcinoma with EBV infection has created along the time a paradigm, due to an extensive use of both serologic tests and IHC or ISH, for detection and screening the high risk population, even if in the last 2 decades, the incidence of this type of cancer has decreased with 30%. A study group of 53 archived formalin-fixed paraffin-embedded samples of undifferentiated nasopharyngeal carcinoma have been assessed using standard HE stain and immunohistochemistry for 4 monoclonal antibodies: EBV latent membrane protein 1-LMP1, multidrug resistance protein-MRP3, the inducible isoform of COX - COX2 and the nuclear transcription factor NF-kB, simultaneously with RNA transcript of EBV (EBER) using an in situ hybridization technique.

A number of direct correlations, statistically significant were noticed between: LMP1 and COX-2 ($r = 0.53$, $p = 0.03$), LMP and MRP3 ($r = 0.5$, $p = 0.0004$), LMP and NF-kB ($r = 0.4$, $p = 0.001$). The ISH reaction for EBER transcript was positive in 22.64% of cases. A low direct correlation, statistically significant was recorded between EBER and LMP ($r = 0.31$, $p = 0.03$), EBER and MRP ($r = 0.4$, $p = 0.05$).

Our data suggest that the latent membrane protein of EBV activates the nuclear transcription factor NF-kB, independent of COX-2 overexpression, via a signalling transduction pathway, from viral genome to cellular genome, in highly aggressive tumours. In MRP3 negative neoplastic cells, the NF-kB overexpression is associated with an aggressive potential of the tumour (with an unfavourable prognostic) and an independent relationship between the resistance to chemotherapy and tumour differentiation.

Keywords: Nasopharyngeal Carcinoma; EBV Transcript; Signalling Molecules

Introduction

Nasopharyngeal carcinoma (NPC), a malignant tumour arising from the epithelial lining of the nasopharynx, occurs worldwide, particularly common in Southeast Asia, northern Africa and among Inuits of Alaska [1,2]. The tumour has a multifactorial etiology including viral, environmental and genetic risk factors, but the precise

role of these factors in the development of NPC still remains unknown [3]. More than 90% of the world's population may have an asymptomatic but persistent, life-long infection with EBV [2,4,5]. During acute infection, EBV primarily infects and replicates in the stratified squamous epithelium of the oropharynx [6]. B lymphocytes are the primary target of EBV latent infection, and the entry route of EBV into epithelial cells is unclear [2]. Since its discovery EBV has been associated with cancers originating from both lymphoid and epithelial cells. The association of EBV with epithelial cell tumours, specifically nasopharyngeal carcinoma (NPC) was described in the literature, along with EBV-positive gastric carcinoma (EBV-GC), a wide variety of B-cell-derived lymphoid neoplasms, including Burkitt lymphoma, lymphomas arising in immunocompromised patients and Hodgkin lymphoma [7,8].

The association of NPC with the EBV was clearly proved in WHO type 2 (non-keratinizing carcinoma) and type 3 NPC (undifferentiated carcinoma), but controversial in type 1 NPC (squamous cell carcinoma) [3,4] making NPC the third most frequent virus-associated malignancy in humans [2]. NPC patients frequently present with one or more of nasal symptoms (epistaxis, nasal obstruction, discharge), otologic symptoms (deafness, tinnitus) cranial nerve palsies associated with the superior extension of the tumour, neck masses or symptoms related to distant spread [1]. The tumour is highly radiosensitive, concurrent chemo- and radiotherapy improves overall survival while surgical resection of the residual or recurrent tumour in the nasopharynx is another salvage option [1]. Significant number of patients relapses, particularly when disease is advanced at diagnosis-the most common presentation due to a lack of early symptoms [9,10]. NPC express a restricted set of viral antigens: the EBV nuclear antigen 1 (EBNA-1) and latent membrane proteins (LMP) 1 and 2 [3].

LMP1 was the first EBV latent gene found to be able to transform cell lines and alter the phenotype of cells due to its oncogenic potential, altering many functional properties involved in tumour progression and invasion. Activation of different signal transduction pathways mediates various downstream pathological effects of LMP1 expression, including cell proliferation, anti-apoptosis and metastasis [11]. In NPC cells, NF-kB plays a critical role in LMP1 mediated signal transduction; LMP1 activate and regulate NF-kB's activity, and NF-kB activation via LMP1 leads to the expression of other related genes, which take part in many biological processes and play critical roles in LMP1 mediated tumorigenesis in NPC [12].

Cyclooxygenase-2 (COX-2), an inducible form of COX, is over expressed in various tumours, raising the possibility of a role for COX-2 in carcinogenesis; it also contributes to angiogenesis. It has been showed that in LMP1-positive NPCs, COX-2 is frequently expressed, whereas LMP1-negative NPCs rarely express the enzyme [13]. Among EBV genes expressed in NPC, EBV-encoded non-polyadenylated RNAs (EBERs) are the most abundant transcripts of EBV in latently infected cells of NPC; although their role remains still unclear, EBERs are believed to induce the initial transformation of epithelial cells, thus contributing to oncogenesis of NPC [14]. Our study aims to assess EBV status in NPC and the role of some molecules involved in the signalling transduction pathways of NPC pathogenesis. We attempted to correlate these results with clinical data.

Material and Methods

A study group of 53 cases (age ranging from 7 to 73 years: M = 39, SE = ± 2.4) with nasopharyngeal carcinomas has been selected from 200 cases with various nasopharyngeal tumours in an interval of 9 years.

The study was carried on archived formalin-fixed paraffin-embedded tissue samples using standard HE stain and immunohistochemistry (IHC) for the following monoclonal antibodies: Epstein-Barr virus (EBV) latent membrane protein 1 – LMP1 (clone: C51-4, 1:100, DAKO), multidrug resistance protein - MRP3 (clone: DTX1, 1:50, Novocastra), the inducible isoform of COX - COX2 (clone: CX294, ready to use, DAKO) and the nuclear transcription factor NF-kB (poly, ready to use, Thermo). The IHC method was an indirect bistadial technique with hydro soluble polymerized dextran, according to manufacturer specifications (Dako EnVision Systems).

In situ hybridization technique for Epstein-Barr virus (EBV) detection was done according to the manufacturer kit specifications (Novocastra, UK), using an oligonucleotidic polyadenilated probe. The probe hybridizes to a RNA transcript (EBER), which encodes the EBV genome in the nuclei of the latently infected tumour cells. The correct positive hybridization signal was an intense blue/black nuclear staining of the tumour cells, with homogenous or granular aspect. Descriptive statistics was used for uniform distributed data using mean, median and standard error. Statistical analysis for correlation between parameters has been done using the SSPS-13 test, running under Windows 7. A value of $p < 0.05$ was considered significant.

Results

The clinical data have revealed that 46.6% of cases were in T2 stage of the primary tumour, 39% of cases were in T3 stage and 14.4% of cases in T4 stage; 45% of patients presented unilateral cervical lymphadenopathy, 30.5% of patients presented bilateral cervical lymphadenopathy and 5% of patients presented lymphadenopaties bigger than 6 cm, localized in supraclavicular fossa. The overall TNM stadialization has shown that 79% of patients were in advanced stages of disease (stage III and IV).

The sex ratio M:F was 1.5:1, men being more frequently affected than women. For men, the mean age was 41 years (SE = ± 2.8), with a maximum peak incidence at 35 years. For women the mean age was 39 years (SE = ± 3.7), with a maximum peak incidence at 24 years. These data were within the epidemiologic limits of the Mediterranean area and with a relatively uniform standard distribution.

The classic histopathology investigation has shown 37 cases (70%) of undifferentiated carcinomas (most frequently-one third–of Regaud nodular type) and 15 cases (28%) of squamous carcinomas (Figures 1 & 2). One case had an undifferentiated carcinoma associated with a keratinizing squamous carcinoma. Other microscopic data recorded were tumour necrosis in 4 cases (7.54%), the presence of tumour infiltrating lymphocytes (TIL) in 7 cases (13.2%) and metastases in cervical lymph nodes in 8 cases (15%).

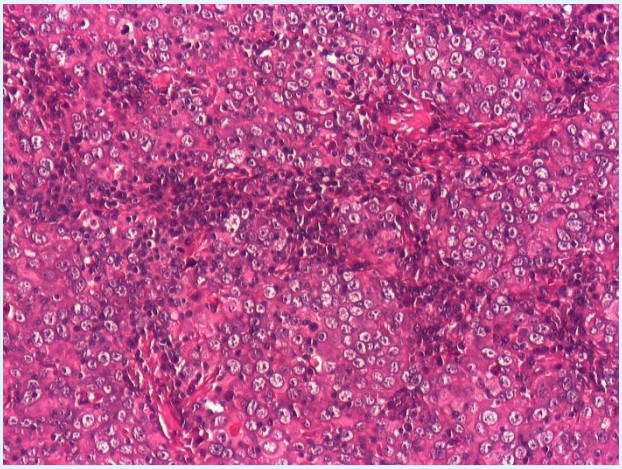

Figure 1: Nodular undifferentiated nasopharyngeal carcinoma (Regaud type), HE, 10x.

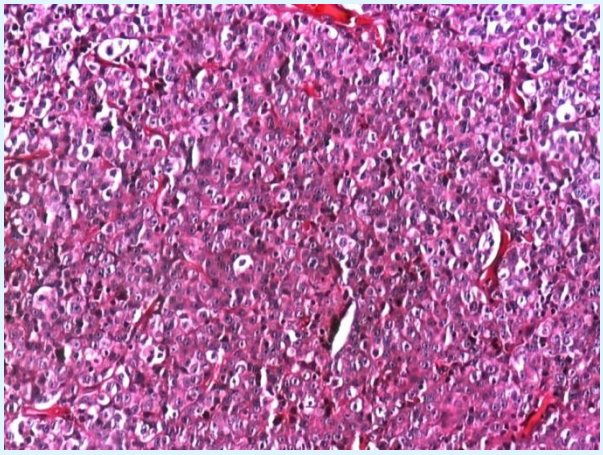

Figure 2: Diffuse undifferentiated nasopharyngeal carcinoma (Schmincke type), HE, 10x.

From the IHC point of view, the multidrug resistance protein (MRP3) was expressed in 15 cases (28.3%), with cytoplasmic granular stain (Figure 3). Nuclear transcription factor NF-kB was also expressed in 15 cases (28.3%), with nuclear and cytoplasmic stain (Figure 4). COX-2 was positive in the cytoplasm of tumour cells (Figure 5) in 9 cases (17%). These 3 markers did not correlate to each other or there was no statistical significance [MRP and COX-2 ($r = 0.24$, $p = 0.12$), NF-kB and COX-2 ($r = 0.14$, $p = 0.19$), MRP3 and NF-kB ($r = 0.53$, $p = 0.79$)]. The latent membrane protein (LMP1) of EBV was expressed in 4 cases (7.54%) with perinuclear granular stain or cytoplasmic clusters (Figure 6). There were some direct correlations statistically significant between LMP1 and COX-2 ($r = 0.53$, $p = 0.03$), LMP1 and MRP3 ($r = 0.5$, $p = 0.0004$) and between LMP1 and NF-kB ($r = 0.4$, $p = 0.001$).

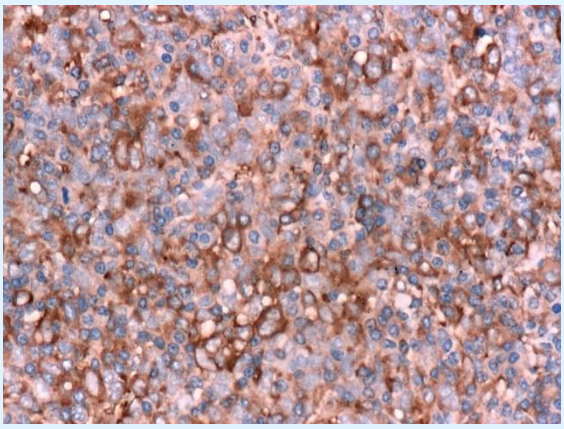

Figure 3: MRP3 positive focally in NPC, IHC, 20x.

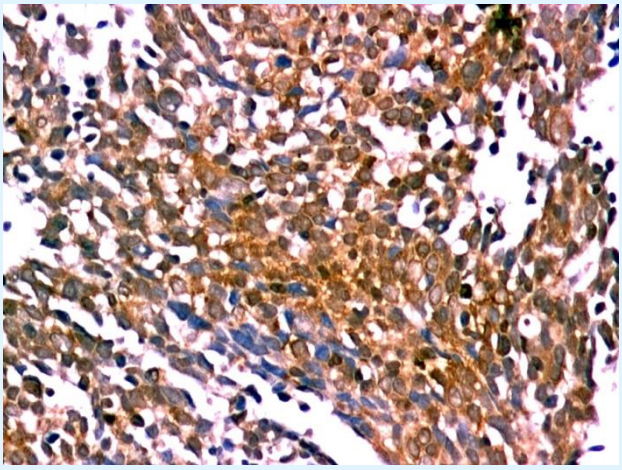

Figure 4: NF-kB positive diffusely in NPC, IHC, 20x.

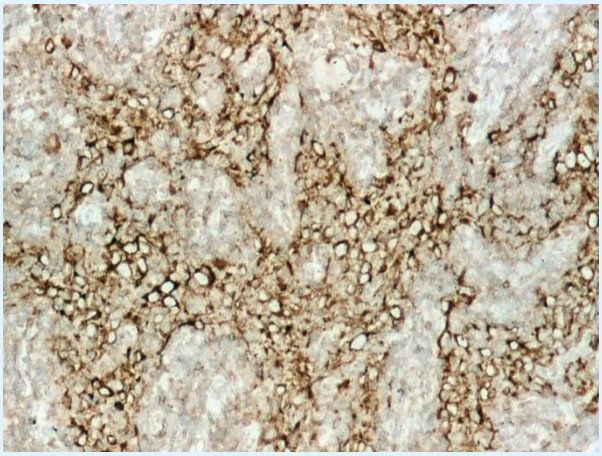

Figure 5: COX-2 positive in NPC, IHC, 20x.

EBV detection in tumour cells nuclei was made using in situ hybridization (ISH) for EBER transcript. Positive signal was noticed only in the nuclei of infected tumour cells (Figures 7 & 8) and was not present in TIL lymphocytes, dendritic cells, vascular endothelium or other stromal cells. No hybridization signal was noticed in the absence of the probe (negative control) and no background reaction was recorded in any case. The ISH variability was qualitatively assessed and numeric quantified for statistical analysis. ISH reactions for EBER transcript were positive in 12 cases (22.64%), with isolated nuclear stain in 4 cases, focally in 7 cases and diffuse in 1 case. The hybridization signal was also noticed in the metastases from the cervical lymph nodes.

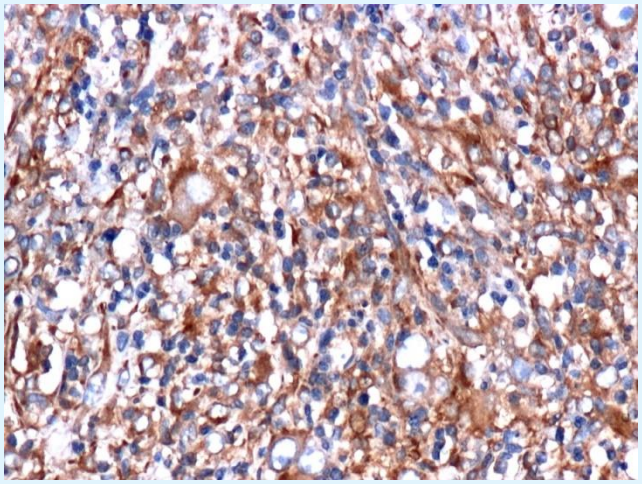

Figure 6: LMP-1 positive in cytoplasm of EBV infected tumour cells, IHC, 20x.

There were two positive direct correlations, statistically significant between EBER and LMP1 (r = 0.31, p = 0.03) and between EBER and MRP3 (r = 0.4, p = 0.05); another correlation was observed between EBER and NF-kB (r = 0.44), but with no statistical significance (p = 0.13).

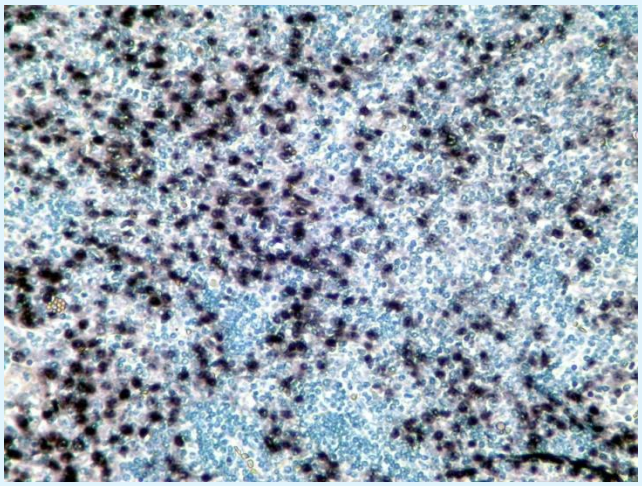

Figure 7: EBV positive in nuclei of infected tumour cells, ISH, 10x.

Discussion

The association of nasopharyngeal carcinoma with EBV infection has created along the time a paradigm, due to an extensive use of both serologic tests and IHC or ISH, for detection and screening the high risk population, even

if in the last 2 decades, the incidence of this type of cancer has decreased with 30% [15].

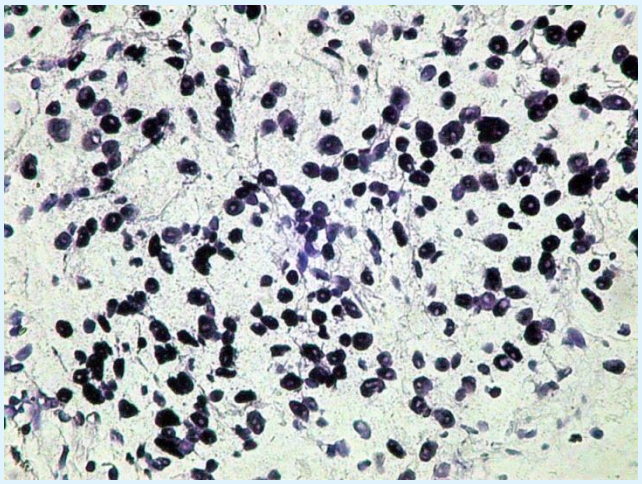

Figure 8: Detail with tumour cells nuclei staining positive for EBV in NPC, ISH, 20x.

The neoplastic process at nasopharyngeal level is seen as a multistep dynamic process, where the EBV infection is a promoter of neoplastic transformation, being also a trigger factor for other types of tumours with monoclonal pattern. Nasopharyngeal carcinomas tend to grow silently until they become unrespectable and have often spread to cervical nodes or distant sites [16].

Epstein-Barr virus-encoded small RNAs (EBERs) (concentrated in the nuclei of latently infected cells being the most abundant viral transcripts) block the activation of dsRNA-dependent eukaryotic initiation factor 2a (eiF-2a) protein kinase DAI. In the absence of EBER, eiF-2a inhibits cellular protein synthesis; therefore, EBER molecules promote host cell survival and viral growth [17,18].

Epstein-Barr virus latent membrane protein 1 (LMP1) activates distinct forms of NF-kB (via two carboxi-terminal regions, CTAR-1 and CTAR-2), producing homodimer complexes; in addition it constitutively activates STAT3 and increases Bcl-3, followed by EGFR overexpression in tumour cells [19]. Also, the signalling transduction pathways from LMP-1 to NF-kB are modulated by EBV encoded micro RNAs, highlighting the role of these sequences in regulating LMP1 downstream signalling to promote cancer development [20]. In this context, the induction of p50/p50/Bcl-3 complexes by LMP1-CTAR1 mediates LMP1-induced EGFR up-regulation, while the formation of these complexes is negatively regulated by the p105 precursor [21].

LMP-1 oncoprotein induces the over expression of COX-2 (an inducible tumour-type of cyclooxygenase, associated with unfavourable prognostic), which significantly correlates to the occurrence of metastases in regional lymph nodes, but does not correlate to the patients age, histopathological type or tumour stage [22].

This observation is supported by other data from the literature, which show that the nucleotidic polymorphism of COX-2 encoding gene is associated with a high risk of metastases of nasopharyngeal carcinoma in cervical lymph nodes [23].

In clinical practice (particularly), COX-2 expression may serve as a marker in predicting the response to radiotherapy in nasopharyngeal carcinoma [24]. According to one study, the low response to chemotherapy of the patients with nasopharyngeal carcinoma is associated to IHC over expression of MRP encoding gene and other proteins, such as: topoisomerase II (Topo II), thymidylate synthase (TS), glutathione-S-transferase (GST-pi) and lung-resistance related protein (LRP) [25]. Also, the recurrent chemical reactivation of EBV promotes genome instability and enhances tumour progression of nasopharyngeal carcinoma cells [26].

Conclusion

Our data suggest that the latent membrane protein of EBV activates the nuclear transcription factor NF-kB, independent of COX-2 overexpression, via a signalling transduction pathway, from viral genome to cellular genome, in highly aggressive tumours. In MRP3 negative neoplastic cells, the NF-kB overexpression is associated with an aggressive potential of the tumour (with an unfavourable prognostic) and an independent relationship between the resistance to chemotherapy and tumour differentiation. Both EBER transcript and LMP correlates to MRP3, being associated to MRP3 over expression and a less sensitivity to chemotherapy.

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